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Bactericidal Action of Daptomycin against Stationary-Phase and Nondividing *Staphylococcus aureus* Cells[▽]

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Most antibiotics with bactericidal activity require that the bacteria be actively dividing to produce rapid killing. However, in many infections, such as endocarditis, prosthetic joint infections, and infected embedded catheters, the bacteria divide slowly or not at all. Daptomycin is a lipopeptide antibiotic with a distinct mechanism of action that targets the cytoplasmic membrane of gram-positive organisms, including *Staphylococcus aureus*. Daptomycin is rapidly bactericidal against exponentially growing bacteria (a 3-log reduction in 60 min). The objectives of this study were to determine if daptomycin is bactericidal against nondividing *S. aureus* and to quantify the extent of the bactericidal activity. In high-inoculum methicillin-sensitive *S. aureus* cultures in stationary phase (10^{10} CFU/ml), daptomycin displayed concentration-dependent bactericidal activity, requiring 32 μg/ml to achieve a 3-log reduction. In a study comparing several antibiotics at 100 μg/ml, daptomycin demonstrated faster bactericidal activity than nafcillin, ciprofloxacin, gentamicin, and vancomycin. In experiments where bacterial cell growth was halted by the metabolic inhibitor carbonyl cyanide *m*-chlorophenylhydrazone or erythromycin, daptomycin (10 μg/ml) achieved the bactericidal end point (a 3-log reduction) within 2 h. In contrast, ciprofloxacin (10 μg/ml) did not produce bactericidal activity. Daptomycin (2 μg/ml) remained bactericidal against cold-arrested *S. aureus*, which was protected from the actions of ciprofloxacin and nafcillin. The data presented here suggest that, in contrast to that of other classes of antibiotics, the bactericidal activity of daptomycin does not require cell division or active metabolism, most likely as a consequence of its direct action on the bacterial membrane.

Most in vitro studies examining the activity of antibiotics have been performed on rapidly dividing planktonic bacterial cultures supplemented with rich growth media. However, in infections, bacteria seldom encounter optimal conditions that allow logarithmic growth. Instead, long periods of limited or arrested growth are normal (20). *Staphylococcus aureus* is known to modulate gene expression to enhance endurance under less-than-optimal growth conditions and survive periods in stationary phase (27). It is likely that stationary-phase or nondividing bacteria are common in many persistent infections (e.g., endocarditis and osteomyelitis) and in biofilm-associated infections (e.g., on catheters, grafts, and foreign bodies) (11, 16). Therefore, antibiotics that are bactericidal under growth-arrested conditions may be beneficial.

The mechanism of action of many bactericidal antibiotics requires ongoing cell activity and cell division for the drugs' killing activity. Such drugs therefore display limited activity against nondividing cultures (4, 14, 15, 17). For example, beta-lactams require ongoing bacterial cell wall synthesis (30), and the action of some quinolones requires ongoing RNA and protein synthesis for bactericidal activity, while others require ongoing cell division (15). Rifampin is one of the few antibiotics identified as retaining bactericidal activity against non-growing bacterial cultures (32).

Daptomycin is a lipopeptide antibiotic that displays rapid bactericidal activity in vitro against gram-positive pathogens, including streptococci, methicillin-resistant *S. aureus*, and vancomycin-resistant enterococci (5, 13, 18, 31). Daptomycin works by disrupting membrane function and causing leakage of essential potassium ions, ultimately leading to loss of membrane potential and cell death (26). Studies of daptomycin using artificial membranes have shown that daptomycin can act directly on the lipid bilayer in the absence of any bacterial protein or other cell surface component. Direct interaction with the bilayer suggests that daptomycin could act independently of growth phase or cellular metabolic activity (23, 24, 26). Bactericidal activity, in the absence of cell proliferation, may be valuable against infections involving slow-growing or growth-arrested gram-positive organisms.

The potency of daptomycin in comparison with other antibiotics was determined against stationary-phase *S. aureus* cultures under growth arrest by nutrient depletion, chemical treatment, and cold temperature.

(Portions of this work were previously presented [45th Intersci. Conf. Antimicrob. Agents Chemother., abstr. E-1743, 2005].)

MATERIALS AND METHODS

Bacterial strain and culture conditions. Methicillin-sensitive *S. aureus* strain ATCC 29213 was used for all of the studies presented here. Bacterial cultures were incubated overnight at 37°C with shaking (200 rpm) in Mueller-Hinton broth supplemented with 50-mg/liter free Ca^{2+} (MHBc) and diluted 1:1,000 into fresh MHBc. To prepare exponential-phase cultures, these diluted cultures were grown in MHBc for 2 h prior to experimentation. To prepare stationary-phase cultures, diluted cultures were grown for 17 to 20 h prior to testing.

Susceptibility testing. MIC determinations were performed in MHBc following Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards) guidelines for broth microdilution MIC assays, except that cultures were incubated at 37°C with shaking at 200 rpm.

Time-kill studies. Bacterial suspension cultures in MHBc were treated with various concentrations of different antibiotics. Time-kill studies were performed

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at 37°C with shaking at 200 rpm, unless otherwise indicated. Culture aliquots (100 μl) were removed at the times shown in the figures, serially diluted in MHB or phosphate-buffered saline (pH 7.4 to 7.6), and then plated on tryptic soy agar fortified with 5% sheep blood and incubated overnight in a 35 to 37°C ambient-air incubator. Cell viability was assessed by enumerating the CFU per milliliter. The lower limit of detection for these studies was 10^3 CFU/ml, and bactericidal activity was defined as a ≥3-log reduction with antibiotic treatment compared with the untreated control at the start of each assay. Undiluted and 1:10-diluted samples were not titrated for the number of CFU per milliliter in order to reduce the effect of drug carryover and avoid misrepresentation of the effect of each compound during the course of the time-kill study.

Time-kill studies of exponential- and stationary-phase *S. aureus*. Exponential-phase *S. aureus* cultures were prepared in MHBc as detailed and then treated with daptomycin at 2 μg/ml. Separately, high cell density stationary-phase cultures of *S. aureus* in MHBc were treated with various concentrations of daptomycin (0, 8, 16, 32, 64, and 128 μg/ml). On the basis of the data generated from the concentration range-finding study, the bactericidal actions of daptomycin and comparator antibiotics were evaluated. Stationary-phase cultures were separately treated with daptomycin, nafcillin, ciprofloxacin, gentamicin, or vancomycin at 100 μg/ml in MHBc. Cell density (the number of CFU per milliliter) was determined at the indicated time points.

Time-kill study of nutrient-depleted, growth-arrested *S. aureus*. The bactericidal activity of daptomycin against exponentially growing, concentrated exponentially growing, stationary, and diluted stationary-phase *S. aureus* was determined in nutrient-depleted (or spent) MHBc medium (depMHBc). depMHBc was prepared by adding *S. aureus* to 400 ml of MHBc and incubating it at 37°C for 46.5 h. Afterward, the cultures were centrifuged (4,648 × *g*) for 10 min at 4°C to collect whole bacterial cells. The supernatant was removed and passed through a 0.45-μm-pore-size filter (Nalgene, Rochester, NY). The calcium concentration in the filtered supernatant was measured with the QuantiChrom Calcium Assay Kit (BioAssay Systems, Hayward, CA) and adjusted to 50 mg/liter.

Stationary-phase cells were prepared as detailed earlier and then centrifuged (6,588 × *g*) for 10 min at room temperature, washed with depMHBc, centrifuged again, and suspended to equal the initial volume in depMHBc. Exponentially growing, concentrated exponentially growing, stationary-phase, and diluted stationary-phase *S. aureus* cells were prepared in depMHBc by standardizing the optical density (OD_{600}) to 3.75 for concentrated exponentially growing and stationary-phase cells and 0.1 for exponentially growing and diluted stationary-phase cells. Bacterial cultures were treated with daptomycin at 10 or 100 μg/ml and assayed at the indicated time points. During each assay, depMHBc sterility was confirmed by incubating a blank sample during the time-kill study.

Time-kill study of chemically growth-arrested *S. aureus*. Exponentially growing cells were grown to an OD_{600} of approximately 0.3. Cultures were divided into three equal portions and left untreated (control) or treated with 10 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) or erythromycin at 2.5 μg/ml. Samples were incubated for 1 h at 37°C while shaking (200 rpm) to allow growth arrest. After the 1-h pretreatment, parallel experiments were performed to test the activity of daptomycin or ciprofloxacin at 10 μg/ml on growth-arrested cultures. Bacterial counts were assayed at various time points.

Time-kill study of *S. aureus* cultures growth arrested by cold temperature. Exponentially growing *S. aureus* cells were chilled in an ice bath for 1 h while shaking (200 rpm) prior to treatment with a concentration of ciprofloxacin, daptomycin, or nafcillin equal to four times the MIC (2 μg/ml). Samples were incubated on ice (0°C) with shaking (200 rpm) during the time course experiment. After 24 h under these conditions, samples were returned to 37°C for 2 h. Numbers of CFU per milliliter were assayed at various time points.

Reagents. Daptomycin was provided by Cubist Pharmaceuticals (Lexington, MA). Other antibiotics, including erythromycin, gentamicin, nafcillin, and vancomycin, were purchased from Sigma (St. Louis, MO), while ciprofloxacin was purchased from U.S. Biological (Swampscott, MA). CCCP and calcium chloride were obtained from Sigma (St. Louis, MO). Culture media, including blood agar plates and MHB, were obtained from bioMérieux (Lombard, IL) and Difco Becton Dickinson (Sparks, MD), respectively.

RESULTS

Susceptibility testing. MIC determinations were performed in MHBc (9). The MICs of the antibiotics used in these studies, determined under growth conditions that would support exponential growth as defined by the Clinical and Laboratory Standards Institute, were as follows: ciprofloxacin, 0.5 μg/ml; daptomycin, 0.5 μg/ml; gentamicin, 0.25 μg/ml; nafcillin, 0.125 μg/ml; vancomycin, 0.5 μg/ml.

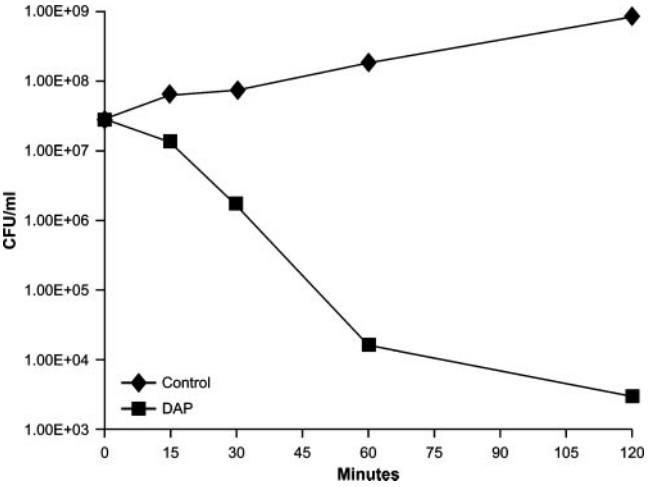

FIG. 1. Daptomycin (2 μg/ml) exhibits rapid bactericidal activity against exponential-phase cultures of methicillin-sensitive *S. aureus*. DAP, daptomycin.

Bactericidal activity of daptomycin against exponentially growing *S. aureus*. As a basis for comparison with experiments in growth-arrested cultures, the effects of daptomycin were evaluated in exponentially growing cultures of *S. aureus*. Daptomycin, at a concentration equal to four times the MIC, was added to cultures to determine the effects on cell viability compared with untreated control cells. At this concentration, daptomycin (2 μg/ml) exhibited rapid bactericidal activity in active cultures of *S. aureus* (as defined by a 3-log reduction in the number of CFU per milliliter) within 60 min (Fig. 1). Untreated bacterial cells divided rapidly, leading to a 20-fold cell density increase (from 5×10^7 to 1×10^9 CFU/ml) during the 120-min-long experiment (Fig. 1).

In order to determine whether daptomycin also had bactericidal activity against stationary-phase organisms, bacteria were grown for at least 17 h to create high inoculum density cultures (1×10^{10} CFU/ml) and then subjected to increasing concentrations of daptomycin in MHBc. In this experiment, *S. aureus* cells appeared to be in stationary phase because untreated cells did not proliferate, as shown by the flat viability curve (Fig. 2). However, in cultures treated with daptomycin, concentration-dependent cell killing was measured. In comparison to exponentially growing *S. aureus*, stationary-phase cultures required higher concentrations of daptomycin (32 μg/ml) to reach the bactericidal end point after 24 h of treatment. This altered concentration response to daptomycin may be due to an altered bacterial physiological (growth) state, culture density, or both.

Bactericidal activities of daptomycin and comparator antibiotics against stationary-phase *S. aureus*. The previous experiments demonstrated that higher concentrations of daptomycin were required for bactericidal activity against stationary-phase *S. aureus* cells compared with exponentially growing cells. Therefore, daptomycin at 100 μg/ml and similarly high concentrations of comparator antibiotics were examined for activ-

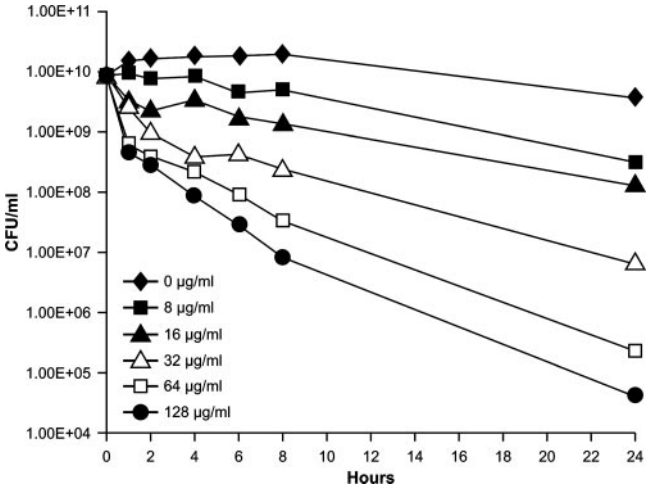

FIG. 2. Daptomycin displays concentration-dependent bactericidal activity against high inoculum density stationary-phase cultures of *S. aureus*. Prior to exposure to daptomycin, cultures were incubated for >17 h to generate high cell density stationary-phase samples.

ity in high inoculum density stationary-phase cultures in MHBc. Stationary-phase *S. aureus* cultures were left untreated or were treated separately with ciprofloxacin, daptomycin, gentamicin, nafcillin, or vancomycin at 100 μg/ml. Under these growth conditions, daptomycin and gentamicin retained bactericidal activity against stationary-phase cultures (Fig. 3). Daptomycin treatment resulted in an immediate and progressive reduction in viability, demonstrating a 3-log reduction in the number of CFU per milliliter after 8 h. Gentamicin required a longer time (24 h) to achieve a bactericidal effect; no change in viability was seen for the first 4 h of exposure. In contrast, under these conditions, ciprofloxacin, nafcillin, and vancomycin did not achieve the bactericidal end point. At the 24-h time point, cultures treated with these three drugs had cell densities equal to those of untreated control cultures (Fig. 3).

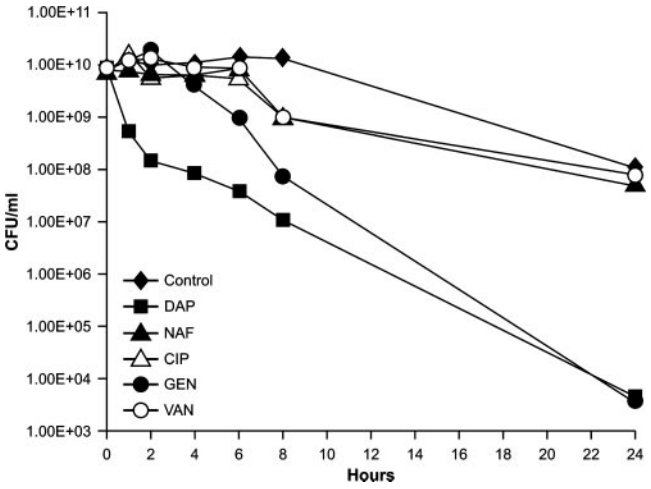

FIG. 3. Effect of exposing stationary-phase *S. aureus* cultures to either daptomycin or a comparator antibiotic at 100 μg/ml. DAP, daptomycin; NAF, nafcillin; CIP, ciprofloxacin; GEN, gentamicin; VAN, vancomycin.

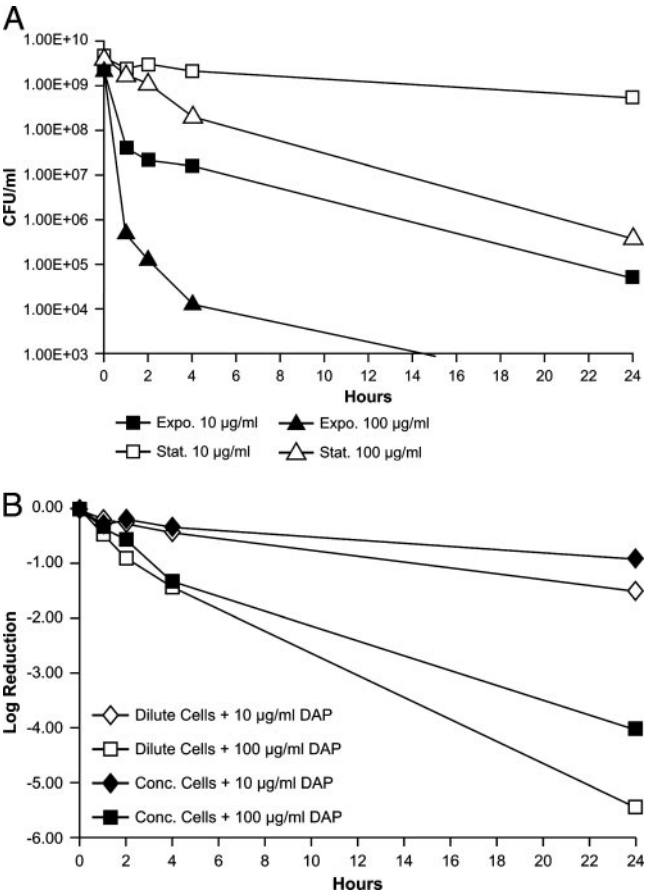

FIG. 4. (A) Effect of cell physiology on the dose response and bactericidal activity of daptomycin against concentrated exponential-phase (Expo.) and stationary-phase (Stat.) *S. aureus*. (B) Log reductions of dilute and concentrated stationary-phase cultures. Cells were suspended in depMHBc and treated with either a high (100-μg/ml) or a low (10-μg/ml) concentration of daptomycin (DAP).

Effects of *S. aureus* physiology on the bactericidal activity of daptomycin. The previous experiment demonstrates that daptomycin retains bactericidal activity against stationary-phase cultures but that elevated levels of the antibiotic are required to achieve the 3-log reduction end point. The requirement for higher drug concentrations could be due to the well-documented inoculum effect (29) or to changes in cellular physiology in stationary-phase cultures. To address this, exponential- and stationary-phase bacterial cultures were prepared at high cell densities (5×10^9 CFU/ml) for treatment with a low (10 μg/ml) or high (100 μg/ml) concentration of daptomycin. The lower daptomycin concentration had a minimal effect on high inoculum density stationary-phase *S. aureus* over the 24-h experiment, while the same concentration was bactericidal against high-density exponentially growing cells over the same time period (Fig. 4A). The high daptomycin concentration (100 μg/ml) had a very rapid bactericidal effect on high-density exponentially growing cells (a >3-log decrease in cell density in 1 h), but in stationary-phase high-density cells, the same concentration was bactericidal only after a prolonged treatment period (Fig. 4A).

To test if altered cell physiology mediates reduced dapto-

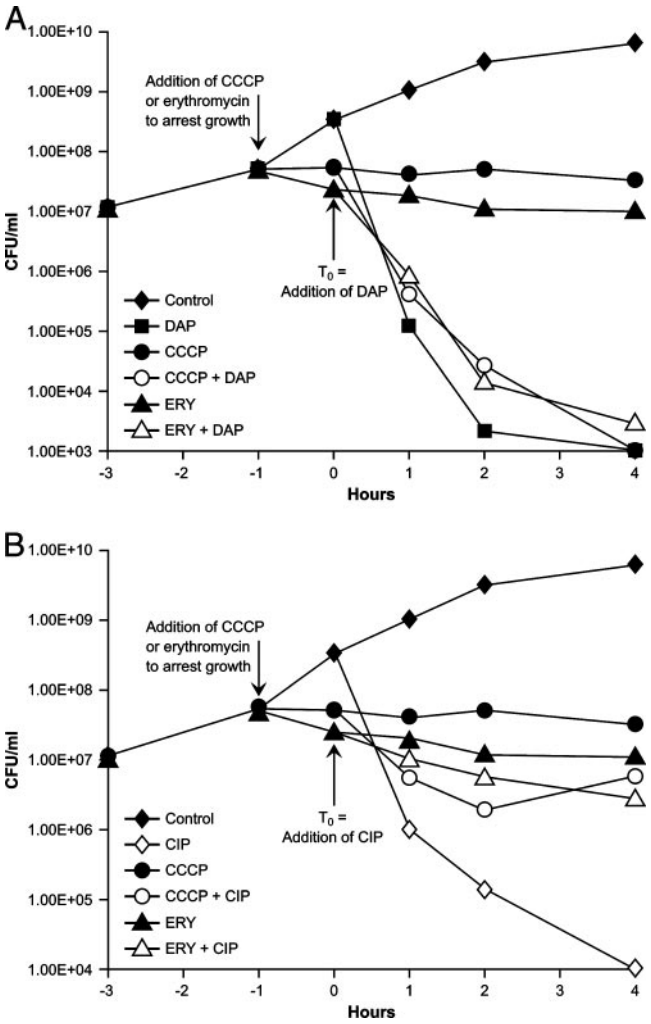

FIG. 5. Effect of daptomycin on chemically arrested *S. aureus*. Exponentially growing cells were chemically arrested by a 1-h pretreatment with CCCP (a proton ionophore) or erythromycin (a protein synthesis inhibitor). Cells were treated with either daptomycin (A) or ciprofloxacin (B) at 10 μg/ml. CIP, ciprofloxacin; DAP, daptomycin; ERY, erythromycin.

mycin susceptibility in stationary-phase *S. aureus*, a culture dilution experiment was performed. Stationary-phase cells were standardized to an OD_{600} of 3.75 and then diluted in depMHBc to an OD_{600} of 0.1. Diluted cultures were treated with a low (10 μg/ml) or high (100 μg/ml) concentration of daptomycin and compared with concentrated stationary-phase cells. The lower daptomycin concentration (10 μg/ml) had no bactericidal effect on concentrated stationary-phase cells over 24 h; however, the diluted cell cultures had some susceptibility (a >1-log decrease in the number of CFU per milliliter) to the lower daptomycin concentration (Fig. 4B). In contrast, the higher daptomycin concentration (100 μg/ml) was bactericidal in both concentrated and diluted *S. aureus* cultures in stationary phase, although the bactericidal effect was more pronounced in diluted cultures. Taken together, these data suggest that under these experimental conditions, *S. aureus* cell physiology influences daptomycin susceptibility more than inoculum density does.

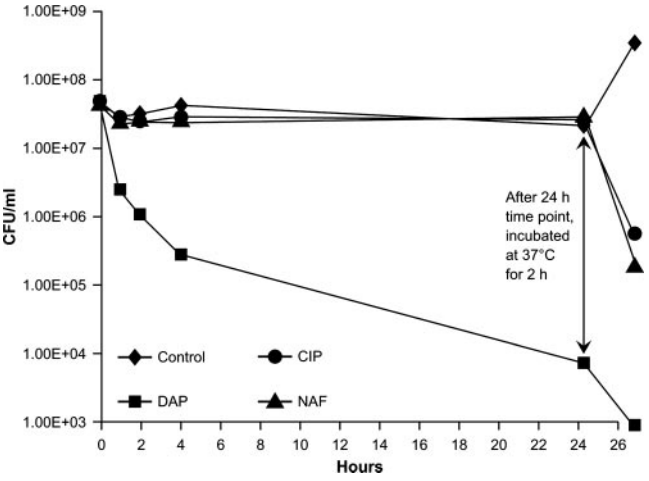

FIG. 6. Bactericidal activities of daptomycin, ciprofloxacin, and nafcillin (each at 2 μg/ml) against *S. aureus* that has been growth arrested by ice bath submersion. After 24 h, bacteria were warmed to 37°C. DAP, daptomycin; CIP, ciprofloxacin; NAF, nafcillin.

Effects of daptomycin and ciprofloxacin on metabolically arrested *S. aureus*. To determine the effect of artificial metabolic arrest on antibiotic susceptibility, exponentially growing cells were treated with a proton ionophore (CCCP) to abrogate cellular energy production or a protein synthesis inhibitor (erythromycin) prior to antibiotic treatment. Untreated control cultures grew almost 3 log units during the 4-h experiment (Fig. 5A and B), while treatment with CCCP or erythromycin stopped the proliferation of bacteria over 24 h (data not shown). Control cultures treated with daptomycin or ciprofloxacin at 10 μg/ml were effectively killed (Fig. 5A and B). Daptomycin also demonstrated a bactericidal effect against *S. aureus* in cultures growth arrested by both CCCP and erythromycin (Fig. 5A). However, CCCP- and erythromycin-mediated metabolic arrest protected *S. aureus* from the bactericidal action of ciprofloxacin (Fig. 5B).

Effects of daptomycin, ciprofloxacin, and nafcillin on *S. aureus* cultures growth arrested by cold temperature. The effects of antibiotics on *S. aureus* cultures growth arrested by cold temperature were also examined. Exponentially growing cultures were chilled in an ice bath prior to treatment with ciprofloxacin, daptomycin, or nafcillin at 2 μg/ml. Time-kill curves demonstrate that daptomycin remained bactericidal against cold-arrested *S. aureus*, while cold arrest protected the cells against ciprofloxacin or nafcillin (Fig. 6). After 24 h on ice, cultures were incubated at 37°C and each of the three antibiotics was active and effectively demonstrated activity (Fig. 6).

DISCUSSION

Daptomycin is a cyclic lipopeptide antibacterial agent that exhibits rapid, concentration-dependent in vitro bactericidal activity against clinically significant strains of gram-positive pathogens, including streptococci, methicillin-resistant *S. aureus*, and vancomycin-resistant enterococci (5, 13, 18, 31). In the present study, the observed bactericidal activity against stationary-phase *S. aureus* ATCC 29213 is remarkable since most other bactericidal agents interfere with cell division or

processes necessary for cell division and typically have little activity against nondividing cells (30).

The mechanism of action of daptomycin has not been fully elucidated, despite 20 years of study. Early efforts (3) suggested that the drug inhibited an early step in peptidoglycan synthesis, although no particular reaction was identified. Other authors (7) have suggested that daptomycin targets lipoteichoic acid biosynthesis. Subsequently, it has been shown that daptomycin becomes inserted into the bacterial plasma membrane in a calcium-dependent fashion, leading to membrane depolarization, release of intracellular potassium ions, and rapid cell death (1, 26). Since depolarization leads to a loss of transport and other metabolic activity, inhibition of lipoteichoic acid and peptidoglycan biosynthesis is a consequence of the primary mechanism (2, 22).

The ability of daptomycin to kill bacteria whose metabolism has been arrested by chemical treatment (dissipation of proton motive force by CCCP or inhibition of protein synthesis by erythromycin), cold, or nutrient depletion further supports the idea that inhibition of macromolecular synthesis is a secondary component of the bactericidal mechanism of action. Recent studies with artificial membranes (19, 26, 28) have suggested that daptomycin insertion causes positive-curvature strain of the membrane, which would ultimately result in cell leakage (28). Although this process has not been demonstrated to occur in vivo, it is consistent with the ability of daptomycin to kill growth-arrested bacteria.

In the present study, antibiotics with different mechanisms of action were selected as comparators. Ciprofloxacin blocks DNA replication, and nafcillin and vancomycin perturb cell wall synthesis, while gentamicin binds to the 30S ribosomal subunit, causing bacterial mRNA to be misread (30). As would be expected, ciprofloxacin, nafcillin, and vancomycin were not bactericidal against stationary-phase *S. aureus* cultures in all of the growth arrest models tested. Interestingly, gentamicin was bactericidal in stationary-phase cells at the 24-h time point. This may be a result of gentamicin's ability to disrupt the cell membrane as it is transported into the cell prior to acting on bacterial ribosomes (6).

It is well established that a high inoculum density can deter the effectiveness of many antibiotics (29). In the experiments presented here, inoculum effects were observed but appeared to have less of an impact on the effectiveness of daptomycin than did cell physiology or the growth state. The reason for this result is unknown. It is possible, however, that positive-curvature strain in the membrane is increased during bacterial cell division, resulting in more rapid bactericidal activity. In some *S. aureus* strains, the bactericidal activity of oleic acid has differential effects, depending on the phase of growth, that appear to coincide with changes in membrane fluidity (33).

A previous study by Lamp et al. had shown that in the presence of human sera, daptomycin was bactericidal in stationary-phase and exponentially growing isolates in vitro and exhibited statistically significantly faster killing rates than vancomycin against both methicillin-sensitive and methicillin-resistant strains (25). In each case, daptomycin and vancomycin were more effective against exponentially growing cultures than against stationary-phase cultures (25). In the present study, daptomycin had bactericidal activity against stationary-phase *S. aureus* at concentrations (~32 μg/ml) approximately 15 times that which is effective against exponentially growing cells (~2 μg/ml). High concentrations of daptomycin (minimum bactericidal concentration, 32 μg/ml) have shown bactericidal activity against adherent *S. epidermidis* (21) in an adherent-cell biofilm model. Interestingly, many of the genes characterized as assisting in bacterial survival in stationary phase, such as Clp homologues, are expressed by bacteria within biofilms (8); these similarities in physiology may translate into similarities in susceptibility.

In conclusion, the data suggest that the bactericidal activity of daptomycin in vitro does not require cell division or active metabolism, consistent with the proposed mechanism of disrupting membrane function. The activity of daptomycin, at high concentrations, against nongrowing cells might also indicate the alternative possibility that daptomycin possesses two mechanisms of action—one active against exponential-phase cultures at low concentrations (near the standard MIC) and one active against stationary-phase cells and observed only at high concentrations of the drug. We have no evidence that would support such a dual mechanism, but the possibility cannot be ruled out by the studies reported here.

The demonstration of daptomycin's bactericidal effect in stationary-phase bacteria may have implications for the additional in vivo use of this drug. Daptomycin was recently approved by the Food and Drug Administration for the treatment of *S. aureus* bacteremia and right-sided infective endocarditis (10, 12). The limitation of these daptomycin experiments is similar to that of many other in vitro studies; experimental conditions do not necessarily reflect those in human infections. However, these studies are valuable in gaining further understanding regarding the mechanism of its action. The present study suggests that further investigations of daptomycin in infection models involving stationary or slowly growing gram-positive bacteria would be of great interest.

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We are all Cubist employees and own stock options.

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